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Insulin-like growth factor-I promotes nerve regeneration through a nerve graft in an experimental model of facial paralysis

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Abstract

Among the pathological sequelae of facial paralysis is a paralytic eye. Apart from the psychological and aesthetic deficits, facial paralysis if left untreated can lead to dryness, ulceration and eventual blindness. Although numerous restorative microsurgical approaches have been introduced to address the sequelae of this problem, complete restoration of function to denervated facial muscles remains elusive.

Utilizing the rat model of facial paralysis the present research has as an objective to examine a dual treatment approach. Specifically, this study combined the current microsurgical treatment of the cross-facial nerve graft with local administration of insulin-like growth factor I (IGF-I).

The efficacy of this combined approach (cross-facial nerve graft + IGF-I) was assessed in the following ways: (a) behavior measurement of the blink response and (b) histomorphometry light and electron microscopy of the entire nerve graft. These data will help provide insight into the restoration of facial muscle function after trauma and assist in the future development of more potent treatment strategies.

The local administration of IGF-I (50 μg/ml) to the cross-facial nerve graft was found to restore the blink response faster and to strengthen the degree of eye closure. Light microscopy examination revealed that IGF-I significantly enhanced axonal regeneration within a nerve graft (a 22 % increase in the mean number of axons), and increased the mean nerve fiber diameter and myelin thickness. Electron microscopy assessment of the nerve grafts demonstrated that the IGF-I treated grafts possessed a greater density of microtubules, which were evenly distributed within the axoplasm.

Keywords: blink reflex, axon, myelin, osmotic pump, neurotrophic, reinnervation

Abbreviations: ALS: amyotrophic lateral sclerosis; B: buccal branch; BI: blink index; C: cervical branch; CFNG: cross-facial nerve graft; DG: distal nerve graft; DT: distal temporal; FP: facial paralysis; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; M: mandibular branch; mRNA: messenger ribonucleic acid; NIH: National Institute of Health; OOM: orbicularis oculi muscle; PG: proximal nerve graft; PT: proximal temporal; VII-T: temporal branch of the facial nerve; Z: zygomatic branch.

1. Introduction

Facial Paralysis (FP) is a devastating injury that gives rise to both functional and aesthetic deficits. Estimates indicate that over 60,000 cases of FP occur in the United States each year [51,70]. Post-traumatic FP has generally a poor prognosis of recovery unless sophisticated microsurgical treatment is used aggressively at an early stage [50,55,61,52,68]. The many similarities between the human and rat facial nerve and its branches have led to the introduction of successful models amenable to experimental manipulation [60,64–66,27,37], and more specifically the rat model of FP and

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treatment with a cross-facial nerve graft (CFNG) [60,64–66]. Unfortunately, treatment of FP with a CFNG does not produce full recovery of function due to diminishing axonal carrythrough across the two sites of coaptation [65].

It is well understood that the peripheral nervous system is capable of regeneration. The mechanisms guiding peripheral nerve regeneration have been the focus of a great number of studies over the years and the role of many soluble trophic factors has been implicated. Denervated nerves, end organs, neurons and glial cells release these substances.

The role of trophic factors in nerve regeneration was first proposed by Forssman in 1898 [10]. Thirty years later, Cajal [2] suggested that regenerating axons preferentially grew towards the distal stump of a transected nerve. He hypothesized that targetless nerves may release soluble substance(s) that could stimulate and direct regeneration.

Today, a plethora of potential candidate growth factors exist [62] that could be considered for this research model in the treatment of FP. Insulin-like growth factor I (IGF-I) was chosen for this experimental study because of its well documented role in enhancing peripheral nerve regeneration.

IGF-I is a single chain polypeptide consisting of 70 amino acids and three disulfide bridges and is a member of the Insulin gene family that also includes IGF-II and insulin [33]. IGF-I and II and their receptors have been shown to be widely distributed in the CNS [56,57,39,53,36]. IGF-I is primarily synthesized in the liver and baseline serum levels in the rat are typically 0.54 ± 0.02 μg/ml [24]. IGFs, like NGF, undergo retrograde transport in axons [18] and may serve to signal the cell body to synthesize cytoskeletal proteins such as α and β tubulins [8,22,23] and neurofilament proteins [67]. A common biochemical pathway can be hypothesized that when activated could lead to neurite formation.

Ishii and colleagues first demonstrated that physiological concentrations of highly purified IGF-I and II could enhance neurite outgrowth in human neuroblastoma cells [46,21]. Later *in vitro* studies demonstrated that IGFs could induce neurite outgrowth in cultured sensory, sympathetic [47] and motor [3,20,42] neurons, as well as enhance neurite outgrowth and increase myelination [9,48].

It has also been reported that both IGF-I [22] and IGF-II [13] gene expression increase in adult rat muscles following denervation. The IGF mRNA levels however return to baseline following regeneration and reestablishment of synapses. These experiments demonstrated that perhaps a feedback inhibitory mechanism exists in adult muscle; and that an increase in IGF mRNA levels is observed in skeletal muscle following denervation.

Immunohistochemistry studies have demonstrated that IGF-I immunoreactivity is increased in the sciatic nerve after lesion, particularly in Schwann cells, but little or no immunoreactivity is found in fibroblasts [15,16]. In addition, IGF-I immunoreactivity has been reported in myelinated axons of the sciatic nerve and in motor and sensory neurons [17,18]. IGF-I has been described as critical to axonal regeneration by facilitating the proliferation of Schwann cells [54]. Further studies have also reported IGF-I receptors located on the growth cones and along the shaft of the axons [3,18].

There is some data suggesting that IGFs are important in both sensory and motor fiber regeneration. Near and colleagues [41] demonstrated that local infusion of IGF-II significantly enhanced the distance that evoked potentials could be detected in motor fibers. Unfortunately, there were no behavioral or histomorphometric studies performed. IGFs have also been shown to be important for sensory axon regeneration which was assessed by quantitatively using a pinch test [25,13].

There is considerable *in vivo* evidence that supports the hypothesis that IGF-I is a neurotrophic factor critical to nerve regeneration. Hansson and co-workers [15] reported that IGF-I accumulated in the rat sciatic nerve after transection and suggested that IGF-I could be involved in peripheral nerve regeneration. Another study [40] manipulated the microenvironment around the injured sciatic nerve and assessed the effects of IGF-I on regeneration. Following sciatic nerve section in the rat, the proximal nerve stump was inserted into a silicone block with three interconnected chambers (Y-tube). The opposing two chambers contained either IGF-I or vehicle. After four weeks the chambers were harvested and nerve regeneration assessed. Results demonstrated that IGF-I infusion increased the distance to which myelinated fibers regenerated. Furthermore, Lewis and colleagues [35] showed that after sciatic crush injury, the density of innervation of muscle increased with IGF-I as well as functional recovery as measured by gripping ability [6].

To date, there have been no studies examining the effect of IGF-I locally administered to a nerve graft. Some studies have previously examined IGF-I locally administered after a crush or freeze injury. In these studies, IGF-I locally administered around a sciatic nerve crush or freeze injury enhanced the rate of regeneration [25,26]. However, the silicone chamber model used in these studies raised major questions of possible ischemia and pressure injury to the nerve due to the enwrapping silicone chamber.

Kanje and colleagues [26] also demonstrated that IGF-I locally administered to damaged neurons could overcome a cycloheximide-induced block of regeneration. Most recently, local doses of IGF-I near the site of sciatic nerve crush or larger systemic doses protected against the impairment of sensory nerve regeneration in streptozotocin diabetic rats [24]. Also, IGF-I systemically administered reversed or arrested diabetic neuropathy effects on hyperalgesia and nerve crush injury in rats [72].

The current study utilized both morphometry and behavior analysis to assess nerve regeneration and recovery of function. Morphometry enables the assessment of nerve regeneration since motor axons with few exceptions are always myelinated and are responsible for critical functions [15]. Furthermore, behavior analysis of the blink response is crucial in assessing recovery of function.

The present study used the rat model of facial paralysis first established in our laboratory [60,64,65] and examined a

dual treatment approach of reinnervating paralyzed muscle in FP with (a) CFNG and (b) CFNG combined with local administration of IGF-I. Efficacy of IGF-I on axonal regeneration through a graft was determined as follows: (1) Behavioral testing of eye sphincter function by the blink test and (2) axonal morphometric analysis of the nerve graft. Analysis of these data will help address the following questions.

(i) What is the degree and timing of eye sphincter closure when reinnervating the paralyzed OOM with a CFNG and local infusion of IGF-I versus CFNG plus vehicle?

(ii) How many axon fibers regenerate through the nerve graft when treated with CFNG + IGF-I and what is their morphology compared to those fibers found in nerve grafts treated with CFNG + vehicle?

2. Materials and methods

2.1. Quantity of animals

Thirteen adult male Sprague Dawley rats divided into two groups were used in this study. (A) The vehicle-treated group consisted of normal rats that received a unilateral facial nerve axotomy just distal to the stylomastoid foramen. A CFNG was performed and an osmotic pump containing a vehicle (1 mM acetic acid) solution ($N = 3$) was implanted. (B) The IGF-I-treated group consisted of animals that similarly received unilateral facial nerve axotomy, and subsequently were treated with a CFNG plus IGF-I (50 μg/ml) infused locally by a similar pump ($N = 10$).

2.2. Surgical procedures

All animals were anesthetized with 100 mg/kg Ketamine / 10 mg/kg Xylazine (intraperitoneal injection) and operated on with the aid of a surgical operating microscope (Zeiss).

The right facial nerve trunk and its branches were explored and identified through a preauricular incision. The temporal branch of the facial nerve (VII-T) to the orbicularis oculi muscle (OOM) was identified using intraoperative nerve stimulation. The right facial nerve trunk was then transected just distal to the stylomastoid foramen producing a complete right facial paralysis (Fig. 1). Two hemoclips were then placed around the proximal nerve stump to prevent reinnervation. The left saphenous nerve (3 cm in length) was then harvested as a graft and tunneled subcutaneously to the contralateral side. The saphenous nerve graft was then coapted to both the left and right VII-T using 11-0 (Ethicon) sutures with a 30-micron needle (Fig. 1). An ALZET (#2004) osmotic pump (200 μl reservoir volume, releasing 0.25 μl/h over four weeks) fitted with silastic tubing (PE-60) catheter was positioned adjacent to the proximal coaptation of the CFNG and anchored in place with three 11-0 epineurial sutures (Figs. 1,2). Group A rats had their pump filled with a vehicle solution (1 mM acetic acid), while Group B animals had their pumps filled with IGF-I (50 μg/ml). Skin closure was accomplished with 6-0 polypropelene (Prolene) sutures. Nine weeks later, the entire CFNG was explored. The osmotic pump was removed; the entire graft (CFNG) was harvested including the proximal and distal coaptations and processed for light microscopy histomorphometric analysis and electron microscopy qualitative analysis as previously described [27,60,64,65].

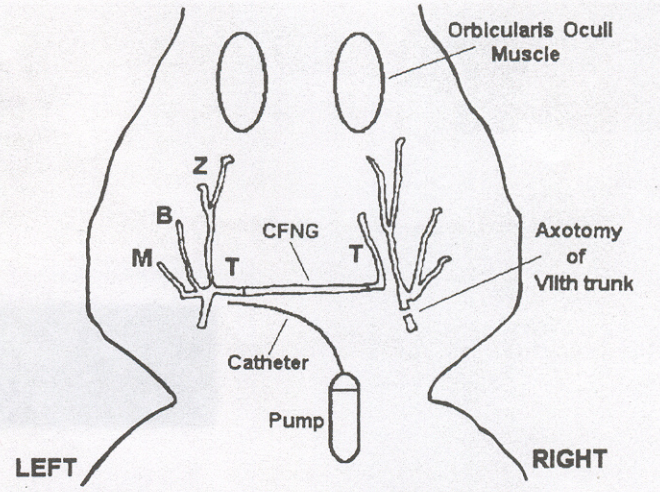

Fig. 1. Diagram of the facial nerve anatomy in the rat illustrating the site of the axotomy, CFNG and placement of the IGF pump catheter.

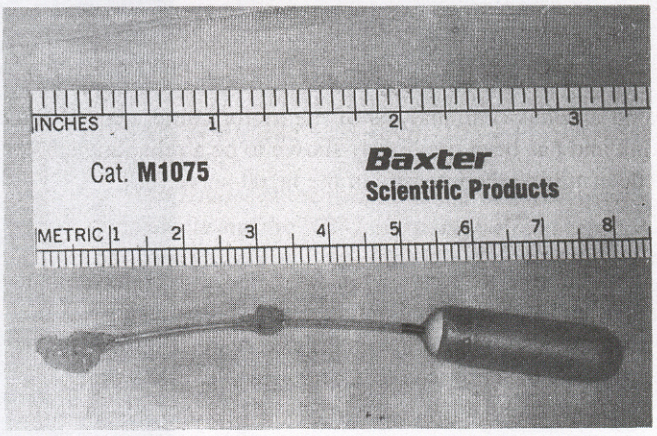

Fig. 2. An Alzet (#2004) osmotic pump attached to a silastic catheter.

2.3. Behavioral evaluation of eye sphincter function – the blink test

Each animal was videotaped for blink response prior to any surgical procedure and at least once a week thereafter, for nine weeks. The blink reflex was evaluated utilizing a custom-designed apparatus (Fig. 3) that delivered a constant 20 ml volume of an air puff to the cornea and periorbital region at a distance of 2 cm [60]. Evaluation of the blink was performed by an independent investigator who graded the blink responses in a double blind manner. A subjective five point blink index grading scale was used which has been previously used both clinically and experimentally [69,27]. Specifically, the blink index scores were as follows: 0 for no blink present – total paralysis, 0.5 for slight contraction of the eye lid(s), 1.0 for observable minimal eye closure (⅓ closure), 1.5 for significant but incomplete eye closure (⅔ clo-

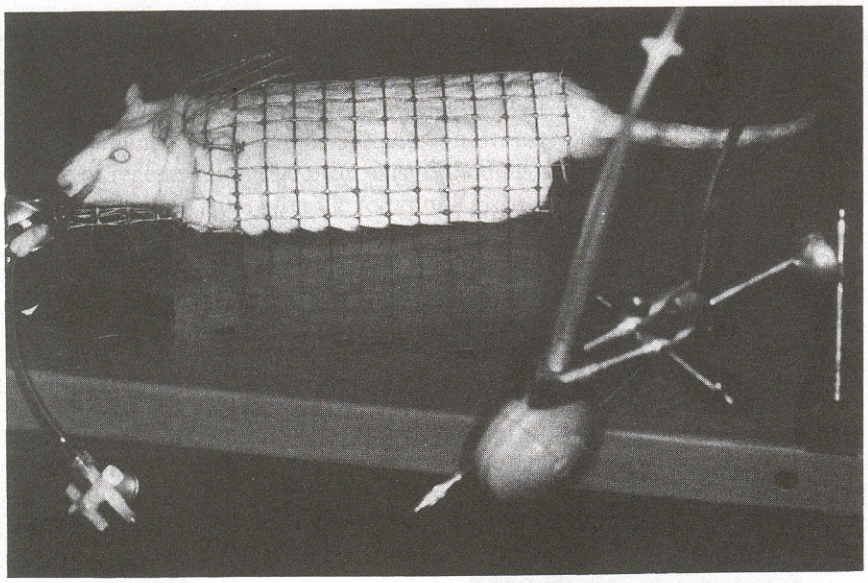

Fig. 3. A rat placed in the blink response testing apparatus.

TABLE 1. Number of days post treatment to which each session corresponds.

Session	*Post treatment day*
1	4
2	10
3	17
4	20
5	24
6	27
7	31
8	35
9	37
10	39
11	41
12	45
13	48
14	53
15	58
16	63
17	67

sure), and 2.0 for complete eye closure. This technique provided a behavioral analysis of the motor component of the blink and has been previously shown to be a reliable method in determining the integrity of the facial nerve [60].

2.4. Data analysis

Quantitative assessment of the CFNG of both vehicle-treated and IGF-I-treated animals was studied using a Zeiss Universal light microscope interfaced with a digitizer tablet (Kurta) and a Sigmascan (3.1) image analysis software. The computational analysis software was developed in our laboratory by L. Dellon in collaboration with Jandel Scientific. The program does assume a circular shape for axons and myelin and has been previously described [27,60,64,65]. All axons displaying irregular or compressed appearance were thus excluded from analysis. Axonal morphometric analysis of each CFNG was carried out as follows: Each CFNG was divided into four nerve specimens (proximal temporal = PT, proximal graft = PG, distal graft = DG, distal temporal = DT; Fig. 4). Each section was analyzed according to the following parameters: (a) Mean number of axons, (b) Mean nerve fiber diameter (μm), (c) Mean axon diameter (μm), (d) Mean myelin thickness (μm). Quantitative assessment of all nerve specimens was based on complete axon counts without sampling. Each axon was traced manually under high magnification (1000×) microscope with a digitizing puck and the quantitative measurements were calculated by the computer. Statistical evaluation of the blink test scores and the histomorphometric profile of each CFNG was performed using paired t-test comparisons.

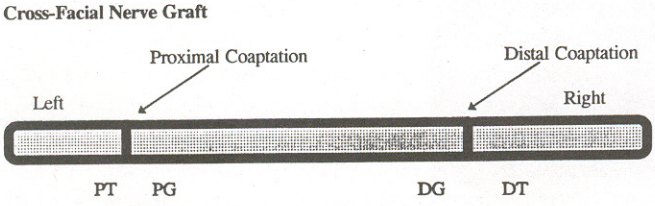

Fig. 4. Diagram of the cross-facial nerve graft divided into four specimens (PT = proximal temporal, PG = proximal graft, DG = distal graft, DT = distal temporal).

2.5. Ethical treatment of animals

All animals were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. This study was approved by the Animal Care and Use Committee of Eastern Virginia Medical School and carried out according to NIH guidelines. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

3. Results

3.1. Behavioral analysis (the blink index)

The key criterion of assessing the efficacy of any treatment for paralysis is functional outcome. In the rat model of facial paralysis, the blink test was utilized. An independent observer unfamiliar with the treatment methodology of each animal graded blink outcome. The functional grading of the blink or the blink index (BI) was used as previously described [69,27]. The blink was tested at least once a week and for a total of 17 sessions (TABLE 1). Exemplary video photographs of the blink response in both vehicle and IGF-I treated animals over time are illustrated in Fig. 5. The mean BI was calculated from eight stimuli (air puffs) for each animal per session (Fig. 6). Assessment of the mean BI between the vehicle-treated rats (CFNG + vehicle) and the IGF-I-

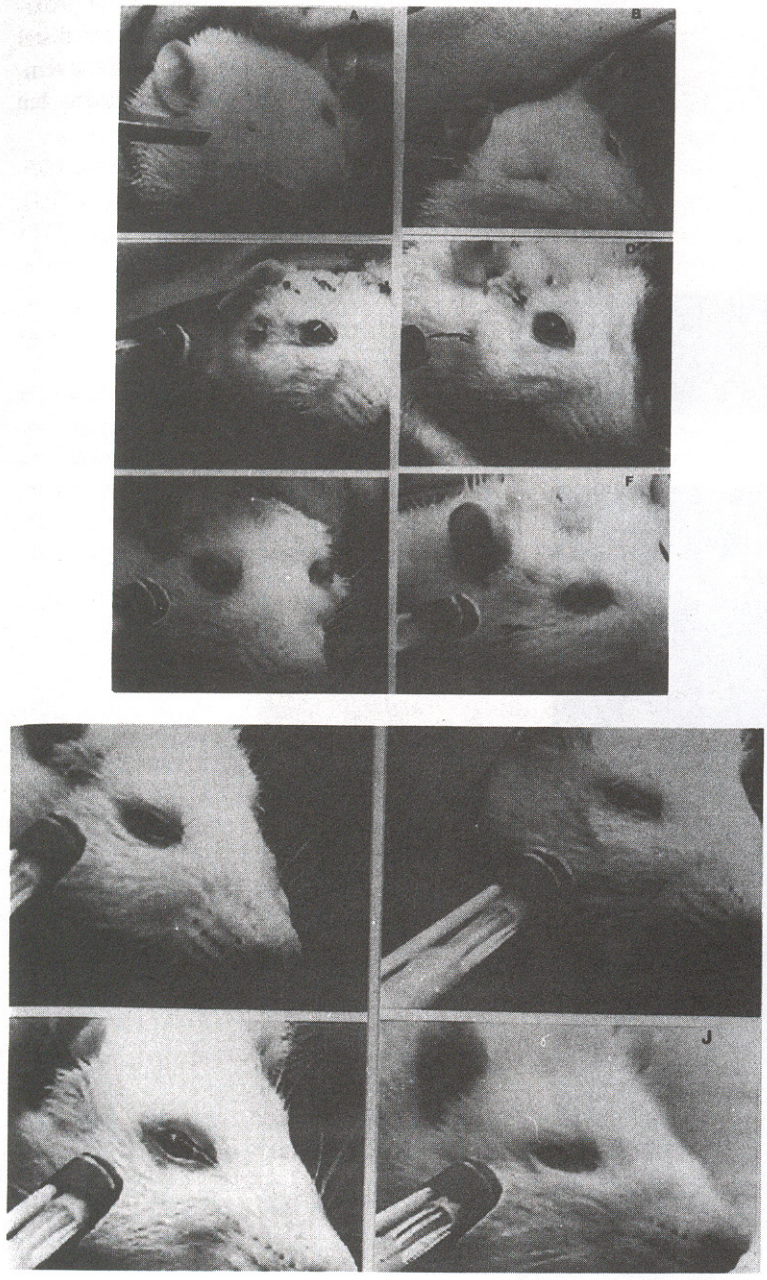

Fig. 5. Photographs of the blink response over time in a vehicle-treated (left) and IGF-I-treated (right) rat. (A,B) = normal preop blink; (C,D) = 2 weeks; (E,F) = 4 weeks; (G,H) = 6 weeks and (I,J) = 8 weeks post treatment.

treated rats (CFNG + IGF-I), using paired t-test comparisons revealed that the IGF-I treated animals had a significantly greater BI than the vehicle-treated animals ($p < 0.01$). More specifically, the BI was observed to significantly differ as early as session 9, which corresponds to 37 days after treatment.

3.2. Qualitative analysis

CFNG specimens were stained with toluidine blue to reveal their axonal and myelin histomorphometry. Transverse 1 μm thick sections of the entire CFNG (PT,PG, DG, DT) are illustrated in Figs. 7–10 (see Fig. 4 for location of nerve biopsy sites). The intraneural organization was maintained in both groups. There was an apparent increase in the number of fibers in the specimens from IGF-I treated animals as well as a decrease of loose intraneural connective tissue.

Ultramicroscopy (Figs. 11a,b, 12a,b) revealed a more random cross-sectional axonal profiles in the IGF-I treated specimens (Fig. 12a) and a striking increase in the number of microtubules present (Fig. 12a, 12b). This abundance of microtubules was evenly distributed throughout the axoplasm (Fig. 12b).

3.3. Quantitative analysis

All specimens were again harvested 9 weeks after treatment. Histomorphometric analyses was performed on all the nerve specimens in both the control and experimental group. The average number of axons found in each of the IGF-I-treated CFNG specimens + SEM was as follows: 193 + 9 (PT), 158 + 8 (PG), 138 + 9 (DG), and 115 + 9 (DT). In addition, the average number of axons found in each of the vehicle-treated CFNG specimens was accordingly: 178 + 5 (PT), 128 + 23 (PG), 103 + 11 (DG), and 67 + 2 (DT).

Assessment of the mean number of axons between the vehicle and the IGF-I rats using paired t-test comparisons revealed that the IGF-I-treated animals showed a significantly greater number of axons versus vehicle-treated rats ($p < 0.001$; tobs = 5.16, df = 11; Fig. 13). Further analysis showed that the IGF-I treated rats contained significantly greater number of axons than vehicle treated rats in the proximal graft (PG; $p < 0.01$; tobs = 3.92, df = 11), distal graft (DG; $p < 0.01$; tobs = 3.82, df = 11) and the distal temporal (DT; $p < 0.001$; tobs = 5.83, df = 11) specimens, but not in the proximal temporal (PT; $p > 0.05$). More specifically, as illustrated in Fig. 13, IGF-I treated animals had 60 % (115.1 versus 192.6 for PT) the number of axons reaching the distal temporal segment of the CFNG as opposed to 38 % (67.3 versus 178 for PT) for the vehicle treated animals. Thus, the IGF-I-treated grafts showed a 22 % increase in the number of axons reaching the distal temporal segment compared to vehicle treated.

The mean nerve fiber diameter (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 5.32 + 0.47 (PT), 4.94 + 0.36 (PG), 4.56 + 0.16 (DG), and 4.54 + 0.20 (DT). In addition, the mean nerve fiber diameter found in each of the vehicle-treated CFNG specimens was accordingly: 4.21 + 0.32 (PT), 4.32 + 0.29 (PG), 4.15 + 0.31 (DG), and 3.43 + 0.09 (DT).

Using paired t-test comparisons overall assessment of the mean nerve fiber diameter in each group showed a significantly larger nerve fiber diameter for the IGF-I treated grafts versus vehicle treated rats ($p < 0.001$; tobs = 4.65, df = 11; Fig. 14). Furthermore, comparison of the mean fiber diame-

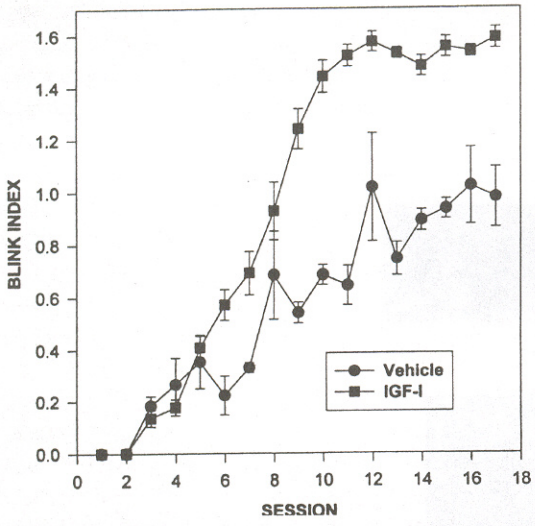

Fig. 6. The mean Blink Index in each group over time (vehicle-treated = CFNG + vehicle, IGF-I treated = CFNG + IGF-I).

ter for the various biopsy sites was as follows: in the proximal temporal (PT; $p < 0.05$; tobs = 2.32, df = 11), distal graft (DG; $p < 0.05$; tobs = 2.53, df = 11) and the distal temporal (DT; $p < 0.001$; tobs = 5.63, df = 11) specimens but not in the proximal graft (PG; $p > 0.05$).

The mean axonal diameter (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 3.09 + 0.12 (PT), 2.99 + 0.07 (PG), 2.90 + 0.06 (DG), and 2.75 + 0.14 (DT). The mean axonal diameter found in each of the vehicle-treated CFNG specimens was accordingly: 2.54 + 0.20 (PT), 2.83 + 0.18 (PG), 2.76 + 0.31 (DG), and 2.77 + 0.26 (DT).

Overall assessment of the mean axonal diameter in the CFNG using paired t-test comparisons showed a significantly larger axonal diameter in IGF-I versus vehicle treated rats ($p < 0.001$; tobs = 3.86, df = 11; Fig. 15). The same relation was found in the proximal temporal (PT; $p < 0.001$; tobs = 4.5, df = 11), and distal graft (DG; $p < 0.05$;

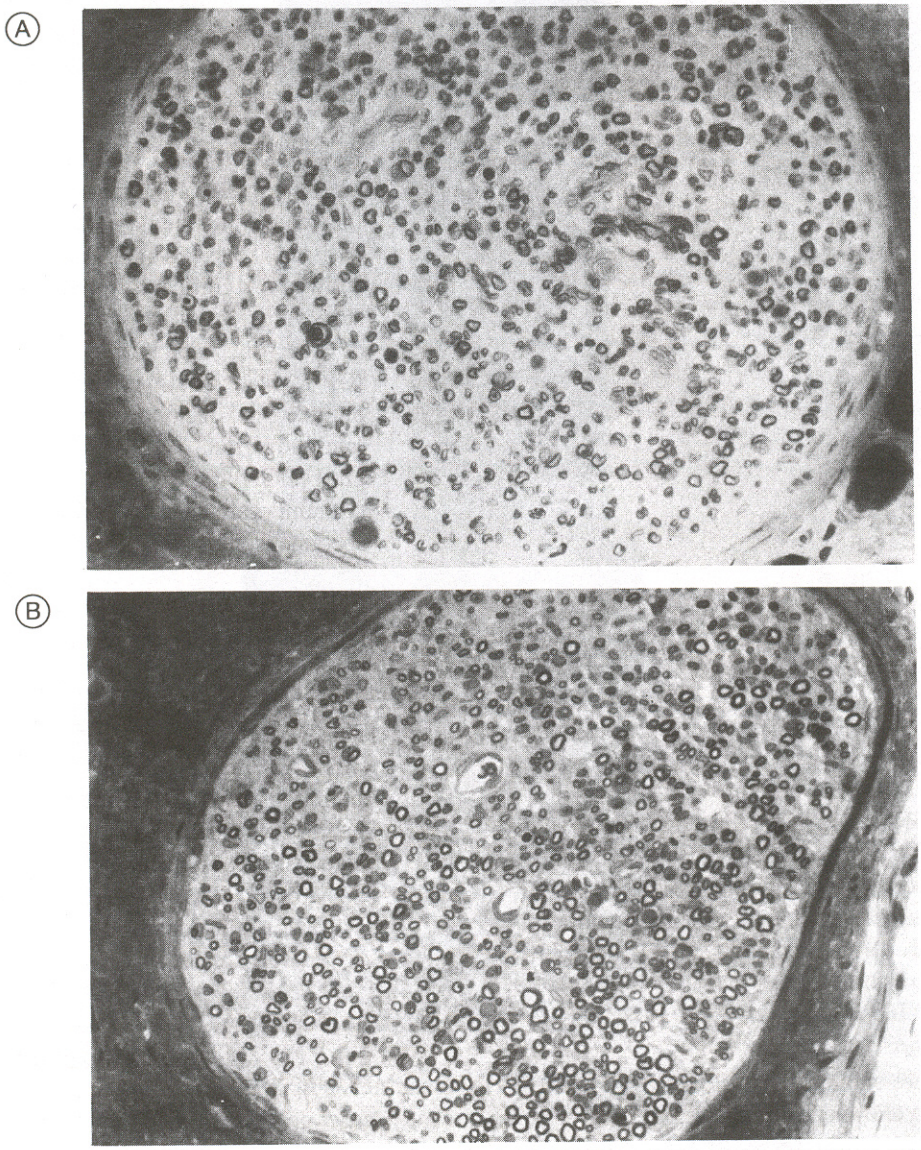

Fig. 7. Micrograph of a transverse 1 μm thick section from a proximal temporal (PT) CFNG specimen stained with 1 % toluidine blue (A. Vehicle-treated, B. IGF-I treated; 400×).

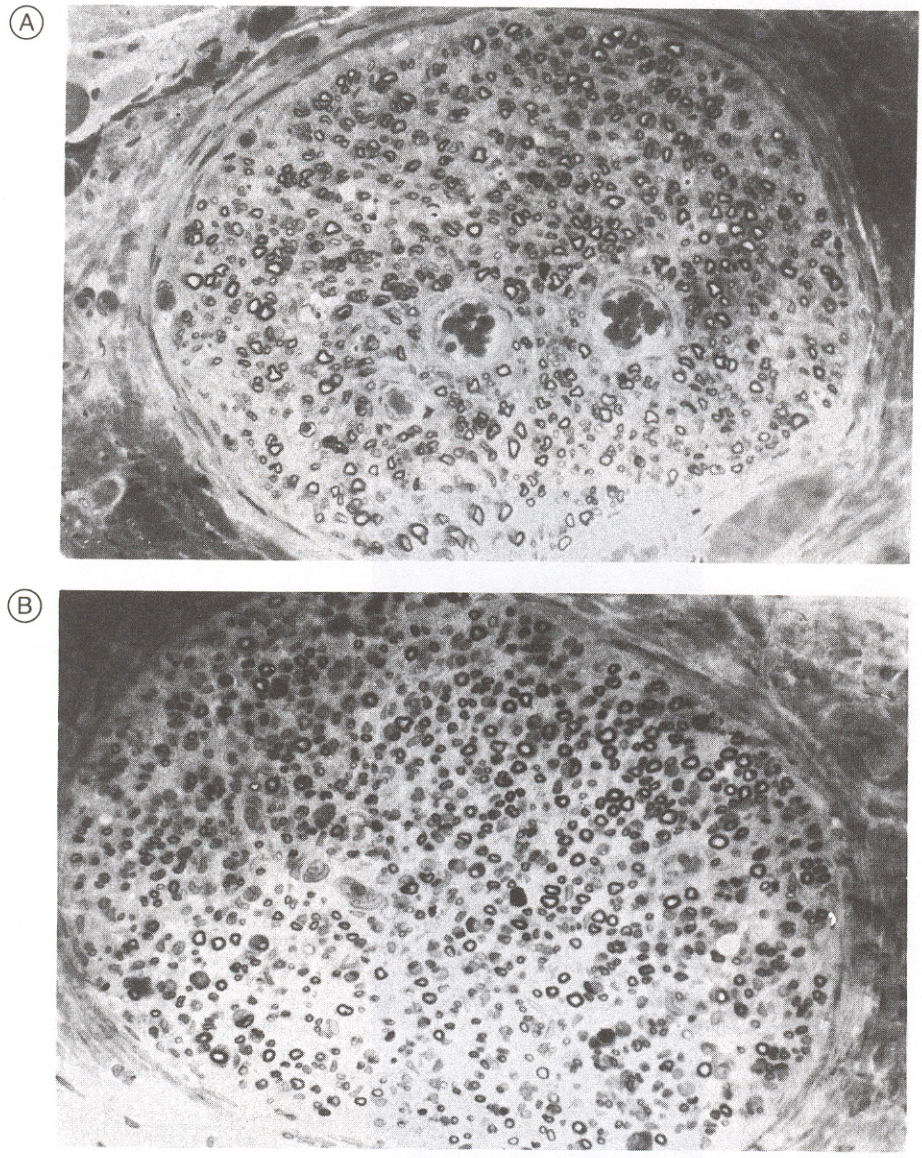

Fig. 8. Micrograph of a transverse 1 μm thick section from a proximal nerve graft (PG) CFNG specimen stained with 1 % toluidine blue (A. Vehicle-treated, B. IGF-I treated; 400×).

tobs = 2.33, df = 11) specimens, but not in the proximal graft (PG; $p > 0.05$) and distal temporal (DT; $p > 0.05$).

The mean myelin thickness (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 0.96 + 0.17 (PT), 0.88 + 0.10 (PG), 0.86 + 0.10 (DG), and 0.79 + 0.11 (DT). The mean myelin thickness found in each of the vehicle-treated CFNG specimens was accordingly: 0.82 + 0.16 (PT), 0.77 + 0.12 (PG), 0.78 + 0.21 (DG), and 0.58 + 0.09 (DT).

The overall assessment of the mean myelin thickness in the CFNG using paired t-test comparisons revealed that the IGF-I treated grafts showed a significantly larger myelin thickness versus nerves in the vehicle treated animals ($p < 0.05$; tobs = 2.22, df = 11; Fig. 16). However, similar statistical comparisons between the various biopsy sites failed to show any significant differences in myelin thickness among the individual specimens ($p > 0.05$).

4. Discussion

The potential role that IGF-I will play in paralysis reversal remains under investigation. In order to enhance functional restoration in post-traumatic facial paralysis, IGF-I was chosen to determine if it had any effect in regeneration through a graft and if so what was the degree of its efficacy.

Outcomes were measured by behavior analysis and histomorphometry. Behavioral results from this study showed that FP animals that were treated with a combination of a CFNG and IGF-I showed a significantly greater degree of functional return (blink index) nine weeks after treatment versus FP animals that received CFNG alone. In addition, testing of the blink index over time yielded an earlier recovery of the blink response for the IGF-I treated rats as early as 37 days after treatment. This sequential behavior observa-

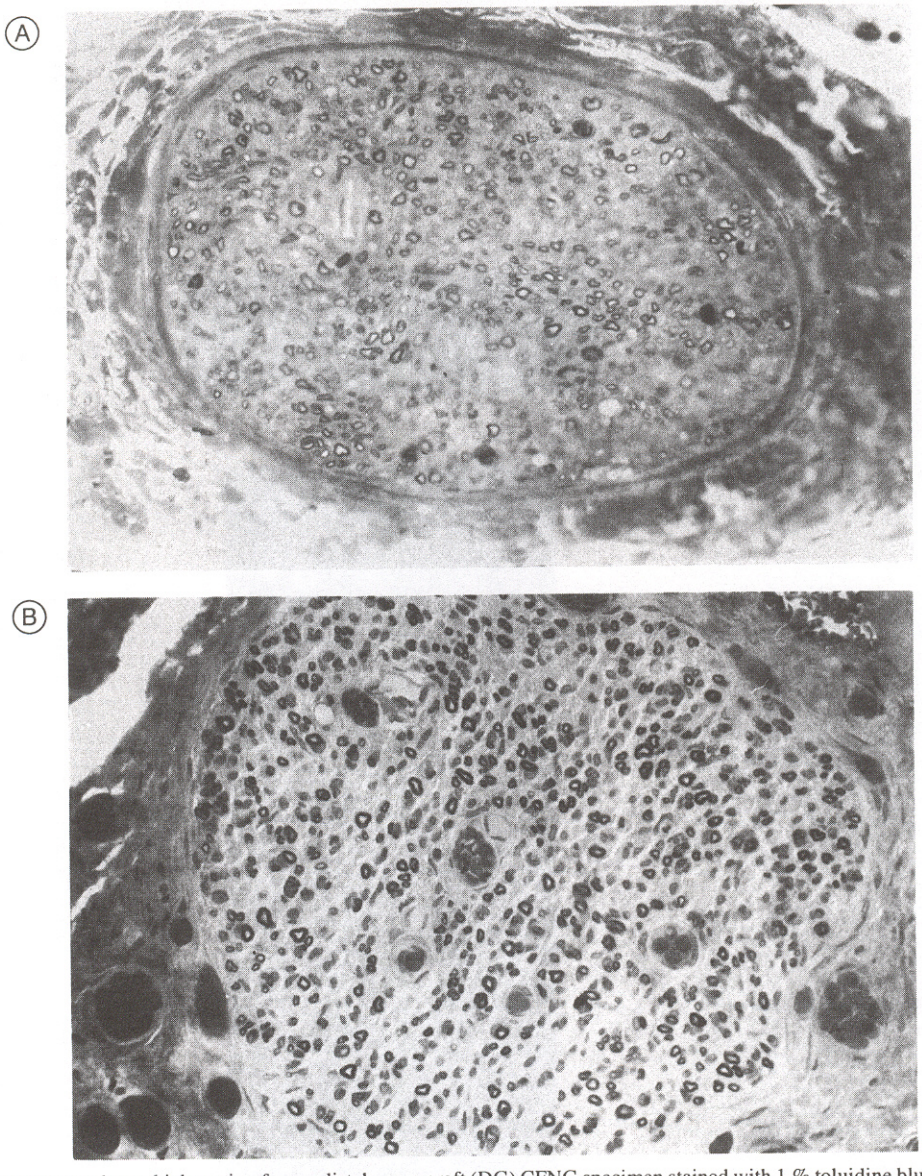

Fig. 9. Micrograph of a transverse 1 μm thick section from a distal nerve graft (DG) CFNG specimen stained with 1 % toluidine blue (A. Vehicle-treated, B. IGF-I treated; 400×).

tion provided functional assessment over time, and thus can identify a critical window of time necessary for IGF-I to have an effect. Thus far, the only available behavioral data on IGF-I has been on sensory fiber regeneration (mouse sciatic crush) by way of a pinch test [25,54,24,13]. However, some of these studies had technical problems with their IGF-I delivery mechanism (silicone chamber), and failed to adequately address the possible additive effects of ischemia and pressure injury in that particular model.

Histomorphometric analysis of the number of axons within the entire CFNG revealed an overall greater number of axons present in the IGF-I treated (CFNG + IGF-I) animals compared to the vehicle (CFNG + vehicle) treated animals. This significant difference in number of axons was found to be greatest in the distal temporal (DT) specimens where IGF-I-treated animals showed a 22 % increase in number of axons, compared to vehicle-treated animals. Thus, it is apparent that IGF-I has played a role in facilitating the carrythrough of axons across the graft conduit and by doing so facilitating successful passage through the second coaptation site prior to obstructive scar formation.

Similarly, the nerve fiber diameter analysis showed that IGF-treated CFNG specimens displayed significantly larger fiber diameters than controls. Furthermore, IGF-I treated CFNG specimens had both a greater mean axonal diameter and myelin thickness than controls; these findings were statistically significant. This difference in axonal diameter was greater than the myelin thickness (as evident from their o level of significance).

It needs to be emphasized that the specimens were from a nine week graft, which probably was inadequate amount o time for completion of myelin maturation. Recently, severa

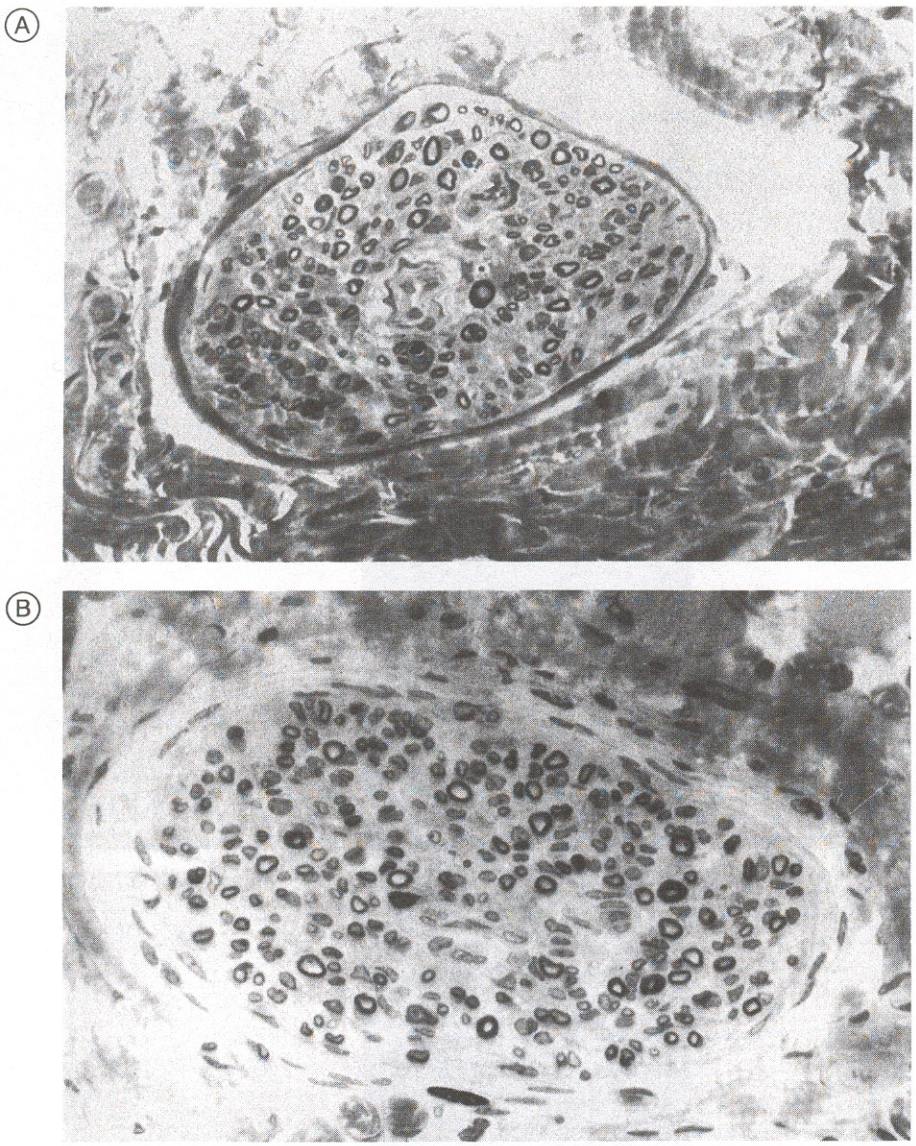

Fig. 10. Micrograph of a transverse 1 μm thick section from a distal temporal (DT) CFNG specimen stained with 1 % toluidine blue (A. Vehicle-treated, B. IGF-1 treated; 400×).

studies have elucidated that IGF-I may play a role in myelination and Schwann cell proliferation [9,48]. Feldman and colleagues [7] studied the role of IGF-I in cultured DRG neurons. They reported that IGF-I promoted differentiation of Schwann cells and their migration to axons. In addition, this study reported that IGF-I promoted myelination independent of its effect on neuronal and Schwann cell survival. Cheng and co-workers [5] described that IGF-I may enhance Schwann cell axonal contact by increasing cell-cell attachment and specifically increase Schwann cell expression of E-cadherin and other adhesion molecules which then leads to long term myelination.

The current study showed, in addition, that in the IGF-I treated nerve grafts the mean axonal diameter was significantly greater. Certainly the outgrowth rate of the IGF-I treated nerve grafts was similar to the slow rate of axoplasmic transport (< 2 mm/day) and thus correspond to the transport of cytoskeletal proteins (microtubules and neurofilaments). A recent study [67] demonstrated *in vitro* that neurite outgrowth induced by IGF-I activate and elevate the mRNA levels of the cytoskeletal 68 kDa and 170 kDa neurofilament and microtubule α and β-tubulin mRNA's. Neurite growth during regeneration requires assembly of the cytoskeleton and IGF-I affects the expression of genes which encode prominent structural proteins of axons, such as neurofilaments which are fibrillar structures that are assembled from 68, 170 and 200 kDa neurofilament proteins [32,43,58]. Qualitative analysis of electron micrographs of the IGF-I treated nerve specimens in the present study demonstrated a visibly greater microtubule density within the axoplasm; these results were in agreement with *in vitro* studies [67].

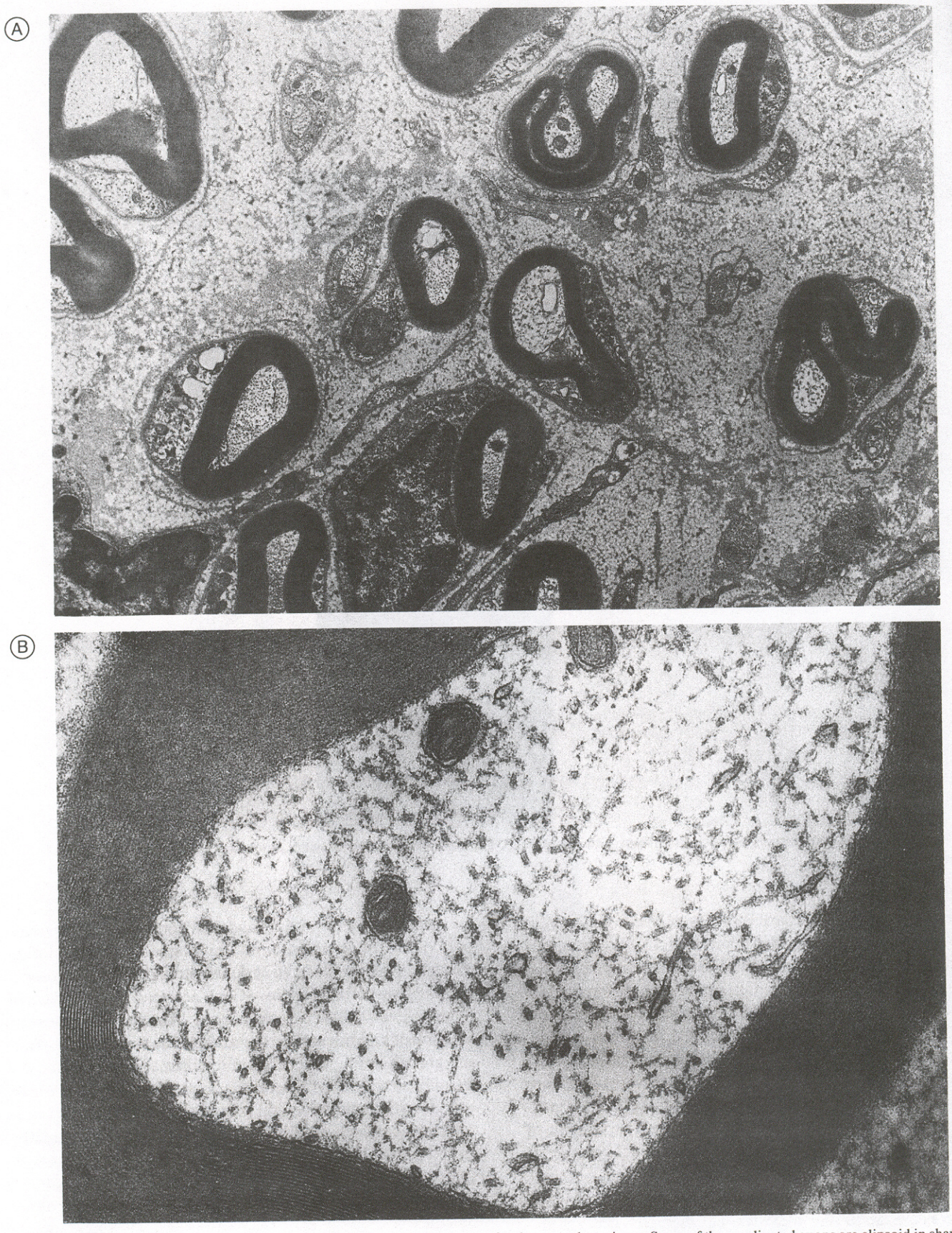

Fig. 11. (A) Electrom micrograph of the vehicle-treated group taken from a proximal temporal specimen. Some of the myelinated axons are elipsoid in shape and possess thin myelin sheaths (5000×). (B) Higher magnification of a myelinated axon from the same specimen. Note the microtubules are sparse and sporadically arranged (25000×).

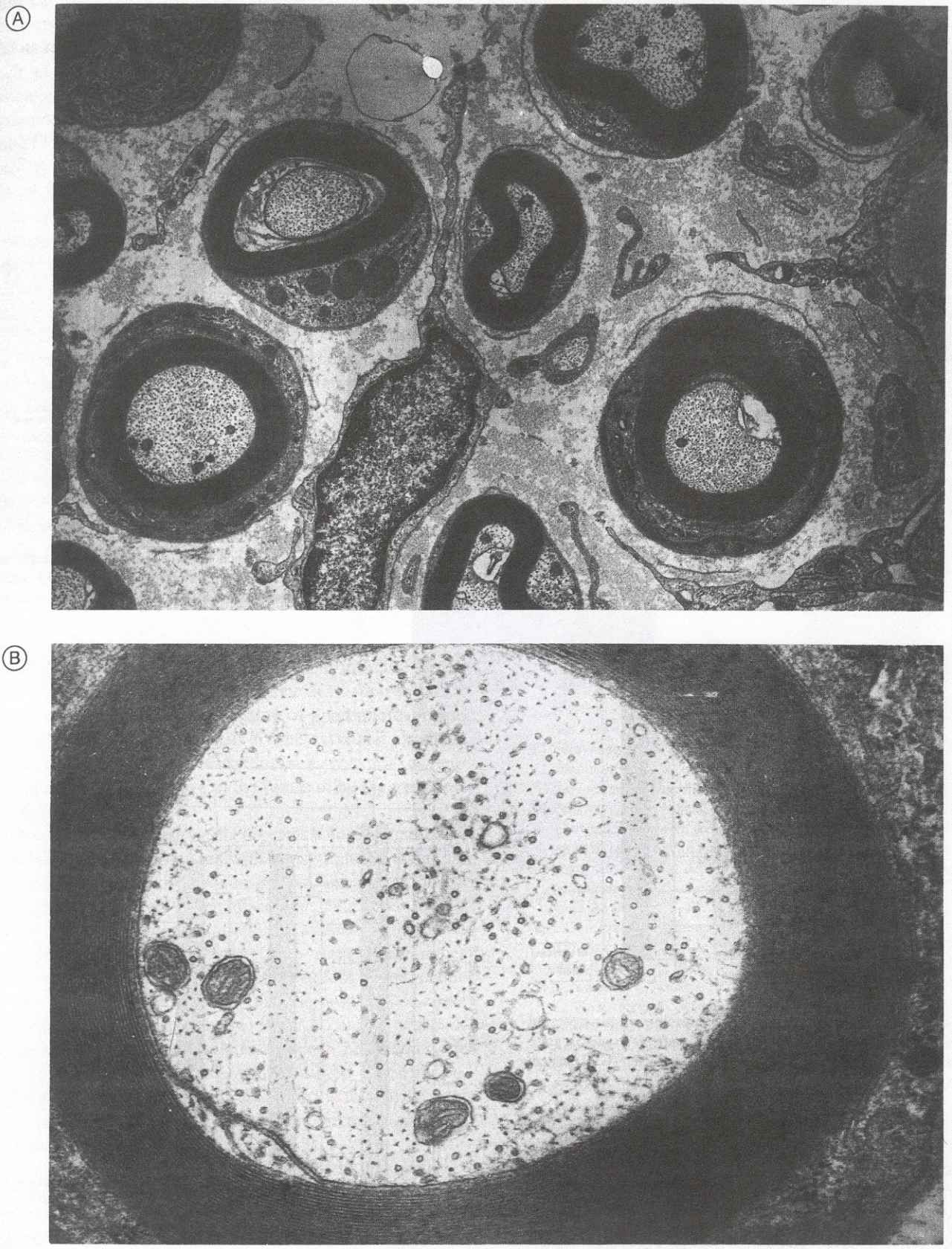

Fig. 12. (A) Electron micrograph of the IGF-I-treated group taken from the proximal temporal specimen. The myelinated axons have a larger diameter and a more spherical profile compared to vehicle. No ultrastructural differences are noted between the Schwann cells of the two groups (5000×).
(B) Higher magnification of a myelinated axon of the same specimen. The cytoskeletal components are more densely packed within the axon. In particular, a greater abundance of microtubules can be observed and these microtubules are homogeneously arranged compared to vehicle (25000×).

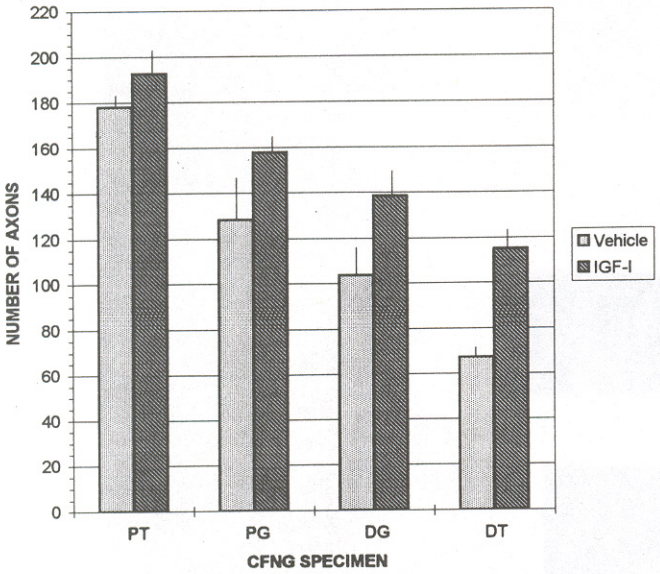

Fig. 13. Mean number of axons in each CFNG specimen versus treatment (PT = proximal temporal-donor, PG = proximal graft, DG = distal graft, DT = distal temporal). Vehicle treated: CFNG + vehicle, IGF-I-treated: CFNG + IGF-I.

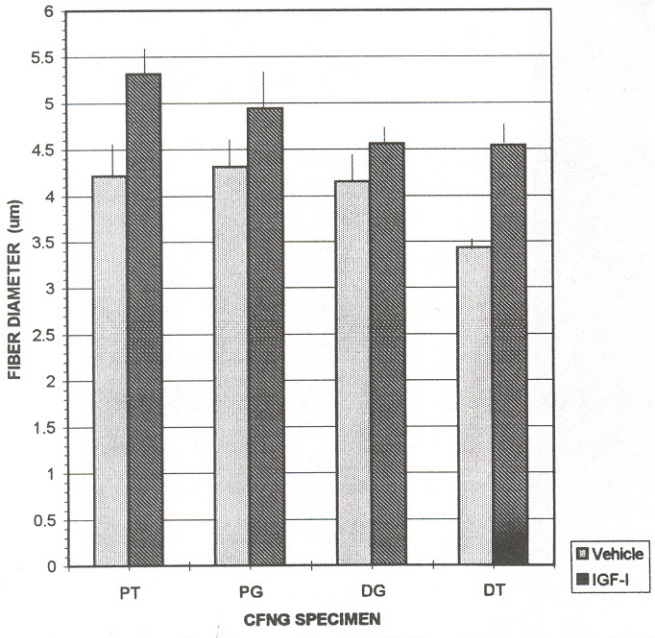

Fig. 14. Mean nerve fiber diameter in each CFNG specimen versus treatment (PT = proximal temporal-donor, PG = proximal graft, DG = distal graft, DT = distal temporal). Vehicle treated: CFNG + vehicle, IGF-I-treated: CFNG + IGF-I.

Facial nerve axotomy initiates a plethora of events including: a) the expression of growth factors and their receptors [30,31,45] b) microglial proliferation and activation and synthesis of various cytokines [11]) and c) astrocyte hypertrophy synthesis of glial fibrillary acidic protein (GFAP) mRNA and GFAP protein, [63] followed by a display of GFAP-positive processes [14]. Recently it was reported that these GFAP-positive astrocytes produce IGF-I during toxin-

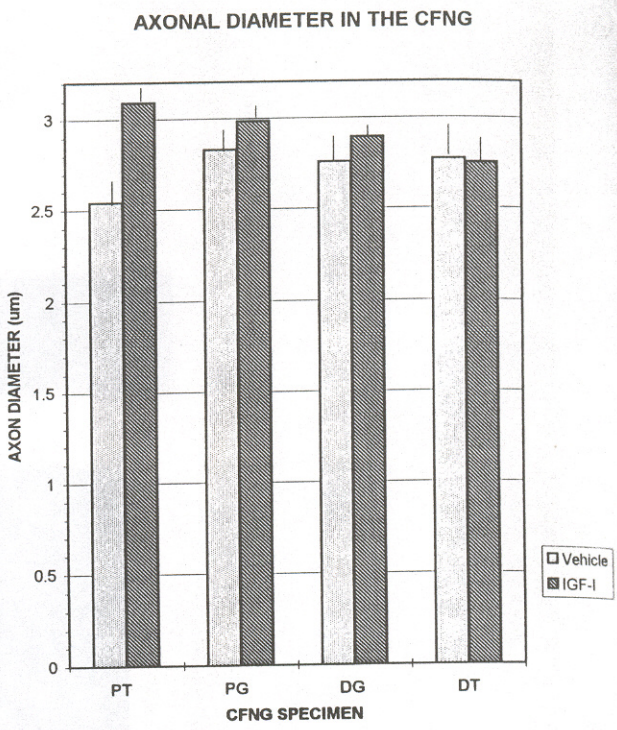

Fig. 15. Mean axonal diameter in each CFNG specimen versus treatment (PT = proximal temporal-donor, PG = proximal graft, DG = distal graft, DT = distal temporal). Vehicle treated: CFNG + vehicle, IGF-I-treated: CFNG + IGF-I.

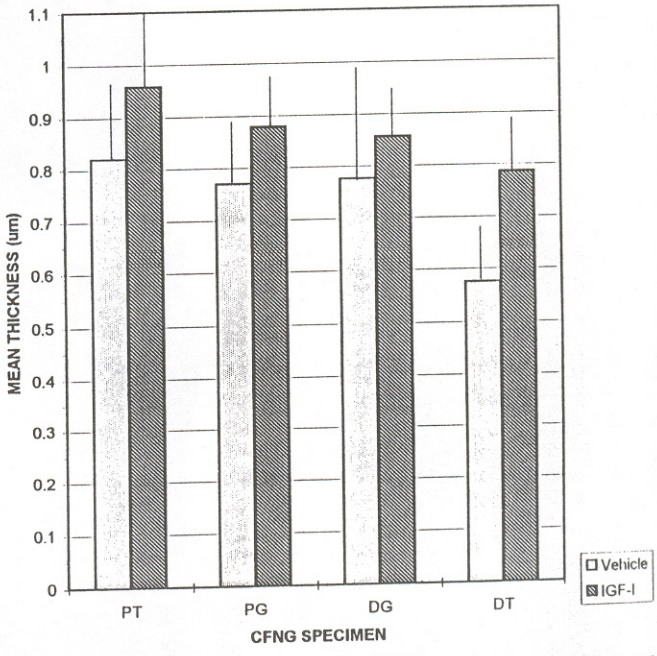

Fig. 16. Mean myelin thickness in each CFNG specimen versus treatment (PT = proximal temporal-donor, PG = proximal graft, DG = distal graft, DT = distal temporal). Vehicle treated: CFNG + vehicle, IGF-I-treated: CFNG + IGF-I.

induced demyelination [29]. Another study reported that facial nerve axotomy induced the gene expression of IGF-I and its receptors [12] as well as the expression of other genes such as CGRP, c-jun and GAP-43 mRNA's [38]. Thus, a distant axonal injury is sufficient to induce this IGF-I astrocytic response. This process is associated with the proliferation of microglia. Their production of cytokines could have a role in the induction of this astrocytic IGF-I response [14,28,59].

In addition, nerve injury causes invading macrophages to produce IGF-I and Schwann cells expressing the IGF-I receptor, which facilitates axonal regeneration [4]. This regeneration can be further augmented by the ability of Schwann cells to secrete IGFBP's which in turn may increase local IGF-I bioavailability from the Schwann cells. However, it has been shown that Schwann cells are not the sole source of polypeptide growth factors in the PNS and trophic factors may also be produced by other cell types present in transected nerves including macrophages, endothelial cells, and fibroblasts [1]. Cheng and co-workers [4] found that macrophages which infiltrate injured nerves and remove tissue debris, serve as another source of IGF-I and also promote Schwann cell proliferation.

The present data on the role of IGF-I in motor regeneration through a graft is encouraging. Clearly, further studies are warranted to define precisely the efficacy of IGF-I in combination with CFNG procedures as treatment strategies for facial paralysis. Furthermore, this study has only explored the role of one of the many neurotrophic factors whose role needs to be defined further in relation to motor nerve regeneration. The combination of good microsurgical techniques in opposing a graft to the proximal or distal nerve, plus the local administration of a 'cocktail' of neurotrophic factors may prove in the future to be the treatment strategy of choice.

The severe functional, aesthetic, psychological and communication deficits associated with injury-induced facial paralysis in addition to the mounting constrains on our health care system have been widely discussed. This paper looked only at the role of one neurotrophin combined with CFNG treatment strategy. In the future gene therapy techniques may provide new perspectives of treatment. The genetic cloning of nerve tissue may alleviate the problems of nerve grafting deficiency in the future. With the aid of neurotrophins, it may soon be possible to approach nerve injury de novo via genetic engineering, with complete regeneration. Without the inherited problems associated with conventional nerve repair, restoration of function may therefore approach normalcy.

In conlusion, insulin-like growth factor-I locally administered to the CFNG was found to enhance the onset of recovery of function as tested by the blink test, as early as 37 days post treatment, and also the degree of functional outcome. Furthermore, IGF-I treated animals showed a 22 % increase in the number of axons reaching the distal end of the graft when compared to the vehicle treated animals. Similarly, IGF-I treated nerves showed an enhanced axon diameter with a greater abundance of microtubules, and a thicker myelin profile in the distal segments of the graft.

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